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Salivary Glyco-sialylation changes monitors oral carcinogenesis

Bhairavi N. Vajaria · Kinjal R. Patel · Rasheedunnisa Begum · Jayendra B. Patel · Franky D. Shah · Geeta M. Joshi · Prabhudas S. Patel

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Abstract Alterations in cell membrane glycosylation play important role in oral carcinogenesis. The present study evaluated salivary sialylation changes *i.e.* total sialic acid (TSA), sialidase activity, linkage specific (α2-3 and α2-6) sialoproteins and sialyl transferase (ST) activity in controls, patients with oral precancerous conditions (OPC) and oral cancer. Subjects enrolled included 100 controls, 50 patients with OPC, 100 oral cancer patients, and 30 post treatment follow-ups. TSA was estimated by spectrophotometric method, sialidase activity by spectrofluorometric assay and linkage specific biotinylated lectins (α2-3: *sambucus nigra* agglutinin and α2-6: *maackia amurensis* agglutinin) were used to detect α-2,3 and α-2,6 STs and sialoproteins by ELISA and dot blot respectively. An increasing trend of salivary TSA/TP ratio, sialidase activity, α2-3 sialoproteins, α-2,3 and α-2,6 ST activities was observed from controls to patients with OPC to oral cancer patients and levels were significantly elevated in oral cancer patients as compared to the controls. Sialidase activity exhibited significant association with metastasis and infiltration. Sialidase activity, TSA/TP ratio, α-2,3 and α-2,6 ST activities were found to be higher in patients with metastasis as compared to patients without metastasis. A progressive increase in TSA/TP ratio, sialidase activity, α2-3 and α2-6 sialoproteins was observed from controls to early to advanced stage of the disease. Sialidase activity, α2-3 and α2-6 sialoproteins and ST activities were found to be decreased in complete responders; while levels were elevated in non-responders. The results documented utility of salivary sialylation endpoints, a non invasive tool in monitoring of oral carcinogenesis.

Keywords Glycosylation · Oral cáncer · Oral precancerous conditions · Saliva · Sialylation · Sialic acid · Sialidase · Sialyltranferase · Sialoproteins

Abbreviations

AJCC	American Joint Committee on Cancer
AUC	Area under curve
CI	Confidence interval
CR	Complete responders
ELISA	Enzyme linked immunosorbent assay
IDV	Integrated density value
LN	Lymph node
OPC	Oral precancerous conditions
MAM	*Maackia amurensis*
MU	Methyl umbelliferone
NR	Non-responders
PNP	*p*-Nitrophenol
PT	Pretreatment
pTNM	Pathological tumor node metastasis
RT	Room temperature
SEM	Standard error of mean
SNA	*Sambucus nigra agglutinin*
ST	Sialyl transferase
TP	Total proteins
TSA	Total sialic acid

B. N. Vajaria · K. R. Patel · J. B. Patel · F. D. Shah · P. S. Patel (✉)
Biochemistry Research Division, The Gujarat Cancer & Research Institute, Asarwa, Ahmedabad 380 016, Gujarat, India
e-mail: prabhudas_p@hotmail.com

R. Begum
Department of Biochemistry, The M. S. University of Baroda, Vadodara, Gujarat, India

G. M. Joshi
Deputy Director, The Gujarat Cancer & Research Institute, Asarwa, Ahmedabad 380 016, Gujarat, India

Introduction

Oral cancer, the leading malignancy in India, is mainly attributed to different forms of tobacco consumption. Apart from tobacco consumption, other etiological factors include viral HPV infection, alcohol consumption, areca nut chewing, pollution *etc.* [1–3]. Oral cancer is one among the few human cancers with a vast potential for prevention and can be cured if treated early enough.

Oral cancer precedes through various preneoplastic stages [4, 5] and the alterations in cell-membrane glycosylation are often associated with neoplastic transformation. Also, cancer being a cellular disease, changes in the cell surface glycoconjugates and enzymes involved in cellular metabolism are of major interest in clinical oncology [6]. Aberrant sialylation in cancer cell is a characteristic feature associated with malignant properties including invasiveness and metastatic potential [7]. Sialic acid (*N*-acetylneuraminic acid) frequently occupies the terminal position on membrane glycoproteins. Cellular sialic acid contents are mainly controlled by sialyl transferases (ST) and sialidases. The amount and types of sialylation of tumor cell membrane depend on the activity of a number of different STs. Sialic acid is linked either through α-2,3 or α-2,6 linkage to subterminal galactose or α-2,8 linkage to another sialic acid forming polysialic acid catalyzed by specific ST. Various STs can be distinguished on the basis of oligosacchide sequence they use as acceptors and anomeric linkage they form with the penultimate sugar residue [8, 9]. Sialidase (neuramindase) enzyme catalyzes the release of terminal sialic acid residue from complex carbohydrate moieties. The major function of sialidase is to hydrolyze glycosidic linkages between sialic acid and glycosyl residue of complex oligosaccharide and glycoconjugates.

Previous studies have reported elevated serum sialic acid levels in patients with oral precancerous conditions (OPC) and oral cancer which suggests its utility in predicting early malignant changes and assessing the spread of invasiveness [10–13]. Earlier studies have observed higher sialidase activity and STs in cancer patients [14–17]. In cancer patients, elevated sialic acid levels are found due to increased serum/tissue sialidase activity. Earlier data from our laboratory have reported increased tissue and serum total sialic acid (TSA), α-2,6 and α-2,3 ST activity and sialoproteins in patients with OPC and oral cancer patients as compared to controls [7]. In furtherance, the present study aimed to translate this data to salivary based biomarkers for its applicability in clinical oncology.

Recently, there is much advancement in salivary based biomarkers for oral cancer detection [18–20]. Oral cancer is one such malignancy where saliva examination can establish its greatest benefit due to its direct contact with the oral cancer lesions. Saliva is an effective non-invasive modality to detect biochemical changes occurring in cancer cells. Various salivary genomics and proteomics biomarkers have been reported in oral cancer; however, salivary glycomics is not much explored. Glycomics has attracted research focus of many scientists in recent years and is now entering into clinical fields [21–23]. Previous studies from our laboratory and also other studies have reported elevated salivary sialic acid in patients with OPC and oral cancer [24–28]. To the best of our knowledge, salivary estimation of α-2,3 and α-2,6 ST activities, α2-3 and α2-6 sialoproteins and sialidase activity have not been reported simultaneously in oral cancer patients and patients with OPC. Simultaneous evaluation of all the sialylation changes would aid in documentation of the molecular alterations in oral cancer progression. We hypothesized that the inclusion of controls and patients with OPC would assist in monitoring early changes occurring during oral carcinogenesis and inclusion of post treatment follow-up patients would help in evaluating treatment response.

Hence, the present study aimed in evaluating salivary TSA, sialidase activity, α-2,3 and α-2,6 STs activities, α2-3 and α2-6 sialoproteins in controls, patients with OPC, oral cancer patients and post treatment follow-ups of oral cancer patients in order to understand the utility of saliva in monitoring changes occurring during various stages of oral carcinogenesis.

Materials and methods

The study was approved by Institutional Review Board of the Gujarat Cancer & Research Institute, Ahmedabad. Due consent was obtained from all the subjects to participate in the study.

Subjects The study subjects included 100 controls that had no major illness in the recent past, 50 patients with OPC and 100 histopathologically proven untreated oral cavity cancer patients. Out of 50 patients with OPC, 39 patients were with oral submucous fibrosis and 11 patients were with oral leukoplakia. Pathological tumor, node and metastasis (pTNM) staging of malignant disease was performed as per American Joint Committee on Cancer (AJCC) norms [29]. The age range was 19–56 years of controls, 16–65 years of patients with OPC and 19–73 years of oral cancer patients. 88 % of oral cancer patients, 100 % of the patients with OPC and 50 % of controls were tobacco habituates. Majority of the tobacco habituates *i.e.* 82 % controls, 88 % patients with OPC and 76 % oral cancer patients were having tobacco chewing habits. Various clinico-pathological characteristics including disease site, histopathology, stage, tumor differentiation and lymph node (LN) metastasis, tumor infiltration were recorded (Table 1). The oral cancer patients were followed up during course of anticancer treatment and total 30 post treatment follow-up samples were obtained for the study. The status of

Table 1 Clinical details of oral cancer patients

Clinical characteristics	Oral cancer patients (*N*=100)
Disease site	
Buccal mucosa	45
Oral tongue	21
Alveolus	08
Gingivo buccal sulcus	04
Retro molar trigone	05
Lip	03
Central arch	03
Hard palate	02
Floor of mouth	01
Multiple sites	08
Histopathology	
Squamous cell carcinoma	97
Verrucous carcinoma	03
Lymph node metastasis	
No	56
Yes	34
Undefined	10
Stage of disease	
I	16
II	16
Early disease (I+II)	32
III	08
IV	54
Advanced disease (III+IV)	62
Undefined	06
Tumor differentiation	
Well	33
Moderate	57
Poor	05
Undefined	05

patients during or after post-treatment was evaluated as described by Therasse *et al.* [30]. The follow-up patients were divided further into complete responders (CR, *N*=25: those who showed good response to anticancer treatment) and non-responders (NR, *N*=5: the patients with stable progressive disease or with no response to anticancer treatment).

Sample collection and processing

Fasting saliva samples were collected between 9.00 and 10.00 a.m. from the subjects to avoid any possible diurnal variations in the study. For collection of saliva, subjects were asked to rinsed their mouth well with water and then expectorate the water. Further, they were asked to spit un-stimulated whole saliva into falcon tube. The tube was kept on ice and saliva was processed immediately after sample collection. For saliva processing, saliva was centrifuged at 2,600 g for 15 min at 4 °C. The supernatant of saliva was collected in different aliquots and protease inhibitors were added [31]. The aliquots were stored at −80 °C until analyzed.

Methods

Estimation of total proteins

Total protein levels from saliva were determined using the Lowry method [32]. The standard curve was prepared using bovine serum albumin (BSA) (Sigma, USA) as standard in range of 10–60 μg.

Estimation of total sialic acid

Total sialic acid was estimated as described by Skoza and Mohos [33] with slight modifications. For the Thiobarbituric acid (TBA) assay of TSA, 0.5 ml supernatant was taken and finally the absorbance was measured spectrophotometrically at 549 nm. The calibration curve was prepared using concentration range of 0–10 μg of *N*-acetylneuraminic acid (Sigma, USA). Concentration of TSA was normalized to total proteins (TP) and the ratio TSA/TP was expressed as TSA (mg/dl)/TP (mg/dl).

Biotinylation of lectins

The α2-6 and α2-3 linkage specific lectins, *sambucus nigra agglutinin* (SNA) and *macckia amurensis agglutinin* (MAM) probes were used for the detection of α-2,6 and α-2,3 linked sialic acid, respectively. Biotinylation of SNA and MAM (Sigma, USA) was performed according to procedure of sulpho-NHS Biotinylation kit (Pierce IL). Biotin conjugated lectins were used for detection of linkage specific sialoproteins and STs activities from saliva samples.

Dot blot for estimation of α2-6 and α2-3 sialoproteins

Estimation of α2-6 and α2-3 sialoproteins was performed by method as described by Shah *et al.* [7] with minor modifications. Briefly, saliva samples equivalent to 50 μg were spotted onto the hybond nitrocellulose membrane. The detection was performed by autoradiography capturing the image on X-ray film. The densitometric analysis of sialoprotein dots was done using gel documentation system (Alpha Innotech Inc., USA). The integrated density value (IDV) *i.e.* sum of all the pixel values after background correction was calculated.

Sialidase assay

Spectrofluorometric method by Potier *et al.* [34] was followed to estimate sialidase activity. Briefly, 10 µl enzyme source (saliva supernatant) was taken for the assay and finally the released fluorescent substrate 4-methyl umbelliferone (MU) was recorded spectrofluorimetrically using exciting light at 365 nm and fluorescence emission at 450 nm. Standard curve was prepared using 4-MU (Sigma, USA) in concentration range of 5 to 150nM. One unit of enzyme activity was defined as µmoles of 4-MU released/min/mg protein. The results were expressed as mU/mg protein.

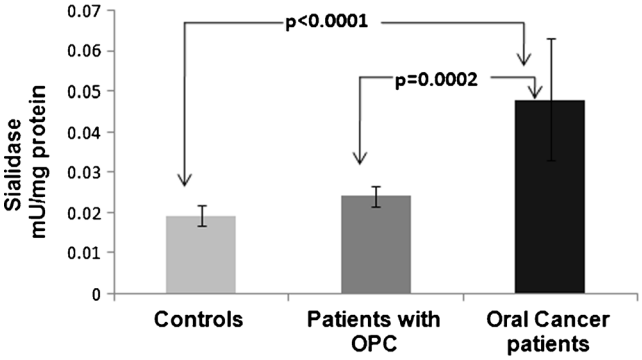

Fig. 2 Comparison of sialidase activity between controls (*N*=30), patients with OPC (*N*=30) and oral cancer patients (*N*=30). OPC: Oral precancerous conditions

ELISA based 96-well solid phase assay for α-2,6 and α-2,3 ST activity

α-2,6 and α-2,3 ST activities were estimated by method as described by Hakomori *et al.* [35], Yeh and Cummings [36], respectively. The absorption was read at 405 nm using an automated microplate reader (Labsystem Multiscan Spectrum, USA). *p*-Nitro phenol (PNP) was used as a standard in range of 20 µM to 200 µM for calibration curve. The unit of enzyme activity (specific activity) was defined as µmoles of PNP liberated /min/mg protein. The results were depicted as mU/mg protein.

Statistical analysis Total sialic acid, total protein, α-2,3 ST and α-2,6 ST activities were estimated from 100 controls, 50 patients with OPC, 100 oral cancer patients and 30 post treatment follow-ups (CR:25 and NR:5). Sialidase activity, α2-3 and α2-6 sialoproteins were estimated from 30 controls, 30 patients with OPC, 30 oral cancer patients and 15 post treatment follow-ups (CR:10 and NR:5). Data were analyzed using statistical package for social science (SPSS Inc. Chicago, IL, USA) software version 17.0. Student's independent '*t*' test was performed to assess the level of significance. Student's paired '*t*' test was performed to compare the levels of sialylation changes between pretreatment and post treatment follow-ups. Pearson's correlation analysis was performed to analyze the correlation between sialylation markers. Univariate analysis was performed to correlate the markers with various clinico-pathological variables. Receiver's Operating Characteristic (ROC) curves were constructed to know the diagnostic efficacy of the markers. The optimal cut off point with highest sensitivity and specificity was determined using Medcalc Software. The values were expressed as Mean ±Standard Error of Mean (SEM). 'p' values less than 0.05 was considered to be statistically significant.

Results

Salivary levels of TSA/TP ratio and sialidase enzyme activity in controls, patients with OPC and oral cancer patients

As depicted in Fig. 1, salivary levels of TSA/TP ratio were found to be significantly higher in patients with OPC and oral

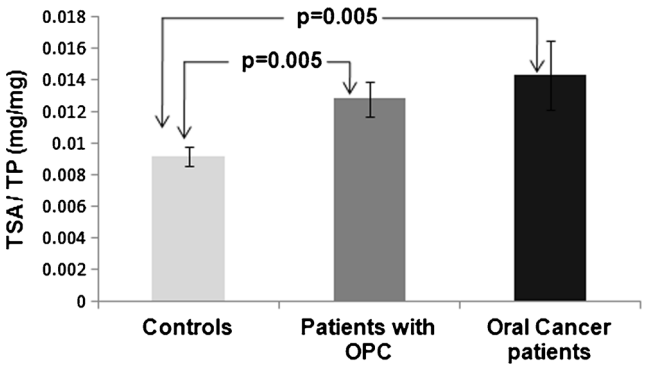

Fig. 1 Comparison of TSA/TP ratio between controls (*N*=100), patients with OPC (*N*=50) and oral cancer patients (*N*=100). *OPC*, Oral precancerous conditions; *TSA*, Total sialic acid, *TP*, Total protein

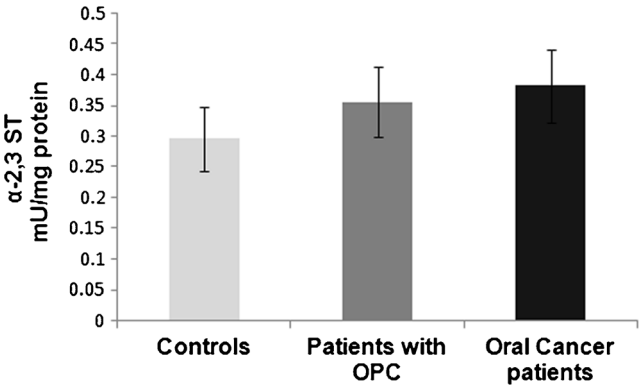

Fig. 3 Comparison of α-2,3 ST activity between controls (*N*=100), patients with OPC (*N*=50) and oral cancer patients (*N*=100). OPC: Oral precancerous conditions; ST: Sialyl transferase

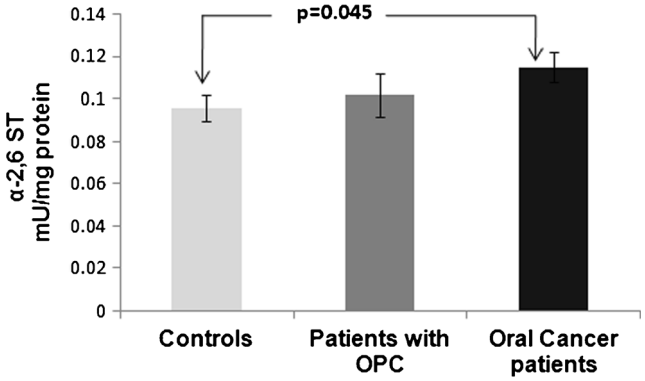

Fig. 4 Comparison of α-2,6 ST activity between controls (N=100), patients with OPC (N=50) and oral cancer patients (N=100). OPC: Oral precancerous conditions; ST: Sialyl transferase

cancer patients as compared to the controls (p=0.005 and p=0.005, respectively). Moreover, an increasing trend was observed from controls to patients with OPC to oral cancer patients. Salivary sialidase activity (Fig. 2) was significantly elevated in oral cancer patients as compared to the controls (p<0.0001) as well as in oral cancer patients as compared to patients with OPC (p=0.0002). Also, the levels were found to be higher in patients with OPC as compared to the controls.

Expression of α-2,6 and α-2,3 STs and sialoproteins

Fig. 3 and Fig. 4 documents a progressive increase in salivary α-2,3 and α-2,6 ST activity from controls to patients with OPC to oral cancer patients. The levels of α-2,6 ST activity were significantly higher in oral cancer patients (p=0.045) as compared to the controls. Fig. 5(a) and Fig. 5(b) represents dot blot pattern of α2-3 sialoproteins and α2-6 sialoproteins respectively. Fig. 5(c) depicts an increasing trend of salivary α-2,3 sialoproteins from controls to patients with OPC to oral cancer patients and the levels were significantly higher in oral cancer patients as compared to the controls (p=0.022). α2-6 sialoproteins (Fig. 5c) revealed only marginal differences between controls, patients with OPC and oral cancer patients.

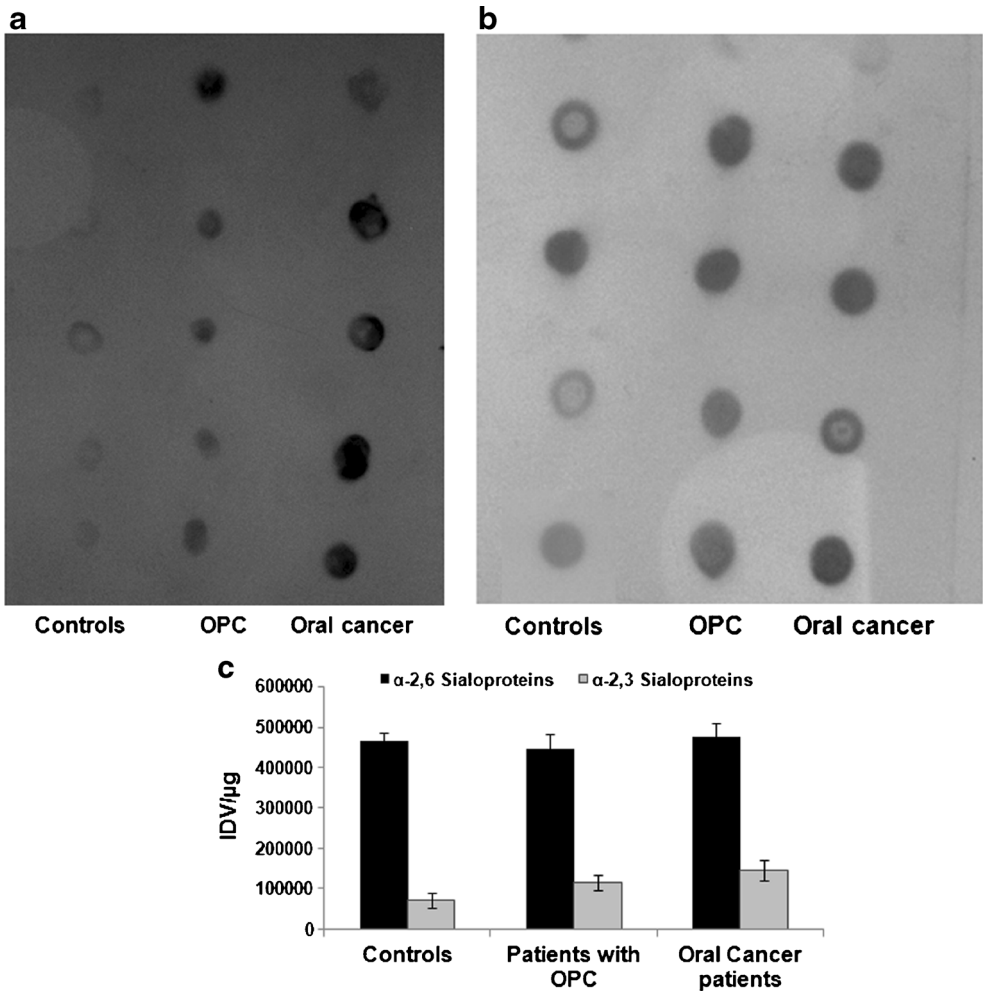

Fig. 5 Comparison of α2-3 and α2-6 sialoproteins between controls (N=30), patients with OPC (N=30) and oral cancer patients (N=30). (**a**) Representative Dot blot pattern for α2-3 sialoproteins (**b**) Representative Dot blot pattern for α2-6 sialoproteins (**c**) Levels of α2-3 sialoproteins and α2-6 sialoproteins in controls, patients with OPC and oral cancer patients. OPC: Oral precancerous conditions

Correlation of salivary sialylation changes with various clinico-pathological parameters

The levels of sialylation changes were compared with various clinico-pathological parameters. Table 2 shows the levels of sialylation markers in early and advanced stage of disease, and in metastatic and non-metastatic patients. As depicted in Table 2, salivary levels of TSA/TP ratio, α-2,3 and α-2,6 ST activities were found to be higher in patients with LN metastasis when compared to patients without LN metastasis. The levels of salivary α2-3 and α2-6 sialoproteins were comparable between patients with metastasis and without LN metastasis. The levels of salivary sialidase activity were found to be significantly higher in patients with metastasis ($p=0.046$) as compared to the patients without metastasis. Univariate analysis depicted significant association of salivary sialidase activity with metastasis ($F=7.078$, $p=0.032$) and infiltration ($F=6.207$, $p=0.042$). An increasing trend of salivary TSA/TP ratio and sialidase activity was observed from controls to early disease then to advanced disease. Further, the levels were significantly elevated in advanced disease ($p<0.0001$) as compared to the controls. The enzyme activities of α-2,3 ST and α-2,6 ST were comparable between controls, early and advanced disease, while an increasing trend of salivary α2-3 and α2-6 sialoproteins was observed from controls to early to advanced malignant disease. The sialylation markers were compared between various grades of tumor differentiation. It was observed that the levels of salivary TSA/TP ratio were found to be higher in moderately as compared to well differentiated tumors, and levels were significant ($p=0.033$). While levels of salivary Sialidase activity, α2-3 and α2-6 Sialoproteins and ST were comparable between well and moderately differentiated tumors. As the number of patients with poorly differentiated tumors was very low, the levels were not compared with poorly differentiated tumors.

ROC curve analysis of salivary sialylation markers

ROC curves analysis was performed to assess the discriminatory efficacy of the markers in distinguishing controls *vs.* oral cancer patients, controls *vs.* patients with OPC and patients with OPC and oral cancer patients. The area under curve (AUC) of above 0.5 suggests valid discriminatory efficacy of the markers. The highest sensitivity and specificity with ideal cut-off, 95 % Confidence interval (CI) and AUC of various sialylation markers are mentioned in Table 3. The results depicted that salivary TSA/TP ratio, sialidase activity and α2-3 sialoproteins significantly distinguished controls and oral cancer patients ($p=0.001$, $p<0.0001$ and $p=0.0015$, respectively). Salivary TSA/TP ratio and α2-3 sialoproteins also discriminated controls and patients with OPC ($p=0.004$

Table 2 Levels of sialylation markers in early and advanced stage of disease, and in metastatic and non-metastatic patients

Markers	Controls (1)	Early disease (2)	Advanced disease (3)	Non-metastatic disease (4)	Metastatic disease (5)
TSA/TP ratio mg/mg	0.00866±0.00065 ($N=100$)	0.0128±0.000101 $p<0.0001$ (1 *vs.* 2) ($N=32$)	0.0156±0.00156 $p<0.0001$ (1 *vs.*3) $p=0.213$ (2 *vs.*3) ($N=62$)	0.0133±0.00132 $p=0.002$ (1 *vs.* 4) ($N=56$)	0.0135±0.00139 $p=0.005$ (1 *vs.* 5) $p=0.942$ (4 vs 5) ($N=34$)
Sialidase mU/mg protein	0.0193±0.0026 ($N=30$)	0.0316±0.0103 $p=0.271$ (1 vs 2) ($N=13$)	0.0461±0.0056 $p<0.0001$ (1 *vs.* 3) $p=0.233$ (2 *vs.* 3) ($N=17$)	0.0367±0.00417 $p=0.001$ (1 *vs.* 4) ($N=20$)	0.0644±0.0183 $p<0.0001$ (1 *vs.* 5) $p=0.046$ (4 *vs.* 5) ($N=10$)
α-2,3 sialoprotein (IDV/μg)	72,379±18,020 ($N=30$)	133,685±34,202 $p=0.093$ (1 *vs.* 2) ($N=13$)	160,290±45,442 $p=0.098$ (1 *vs.* 3) $p=0.646$ (2 *vs.* 3) ($N=17$)	151,345±39,169 $p=0.086$ (1 *vs.* 4) ($N=20$)	96,389±35,423 $p=0.565$ (1 *vs.* 5) $p=0.458$ (4 *vs.* 5) ($N=10$)
α-2,6 sialoprotein (IDV/μg)	464,046±23,874 ($N=30$)	555,398±30,825 $p=0.198$ (1 *vs.* 2) ($N=13$)	585,003±36,221 $p=0.095$ (1 *vs.* 3) $p=0.597$ (2 *vs.* 3) ($N=17$)	473,604±23,874 $p=0.881$ (1 *vs.* 4) ($N=20$)	453,160±49,360 $p=0.853$ (1 *vs.* 5) $p=0.837$ (4 *vs.* 5) ($N=10$)
α-2,3 ST mU/mg protein	0.296±0.0052 ($N=100$)	0.522±0.00164 $p=0.199$ (1 *vs.* 2) ($N=32$)	0.327±0.0679 $p=0.724$ (1 *vs.* 3) $p=0.278$ (2 *vs.* 3) ($N=62$)	0.4058±0.09695 $p=0.828$ (1 *vs.* 4) ($N=56$)	0.4538±0.1401 $p=0.619$ (1 *vs.* 5) $p=0.783$ (4 *vs.* 5) ($N=34$)
α-2,6 mU/mg protein	0.0963±0.0060 ($N=100$)	0.122±0.0164 $p=0.077$ (1 *vs.* 2) ($N=32$)	0.109±0.00853 $p=0.222$ (1 *vs.* 3) $p=0.445$ (2 *vs.* 3) ($N=62$)	0.1126±0.1065 $p=0.853$ (1 *vs.* 4) ($N=56$)	0.1267±0.0142 $p=0.08$ (1 *vs.* 5) $p=0.450$ (4 *vs.* 5) ($N=34$)

Table 3 ROC curve analysis of salivary sialylation markers showing cutoff, AUC, sensitivity and specificity

Groups compared	Saliva	TSA/TP	Sialidase	α2-3 sialoproteins	α2-6 sialoproteins	α-2,3 ST	α-2,6 ST
Controls	Cutoff	0.0062	0.272	2202816	25844824	0.5904	0.0708
vs.	AUC	0.664	0.833	0.754	0.543	0.507	0.588
Oral cancer	Significance	p=0.0001	p<0.0001	p=0.0015	p=NS	p=NS	p=NS
patients	Sensitivity	96.6 %	72.41 %	95.7 %			
	Specificity	32.2 %	80.77 %	47.1 %			
	95 % CI	0.587–0.734	0.708–0.920	0.593–0.876	0.352–0.725	0.420–0.594	0.504–0.667
Controls	Cutoff	0.0113	0.0161	3403080	15793920	0.1043	0.1114
vs.	AUC	0.658	0.640	0.779	0.604	0.594	0.515
patients	Significance	p=0.004	p=0.0764	p=0.0007	p=NS	p=NS	p=NS
with OPC	Sensitivity	57.2 %	80.0 %	70.0 %			
	Specificity	71.6 %	50.0 %	76.5 %			
	95% CI	0.567–0.741	0.493–0.770	0.604–0.902	0.427–0.762	0.492–0.690	0.421–0.608
Patients	Cutoff	0.0056	0.0219	10030392	12858624	0.1922	0.112
with OPC	AUC	0.522	0.749	0.514	0.704	0.602	0.597
vs. oral	Significance	p=NS	p=0.0002	p=NS	p=0.0298	p=NS	p=NS
cancer	Sensitivity		79.3 %		100.0 %		
patients	Specificity		64.0 %		36.8 %		
	95 % CI	0.433–0.610	0.612–0.857	0.351–0.675	0.518–0.852	0.525–0.687	0.518–0.673

and p=0.0007, respectively). Moreover, salivary sialidase activity and α2-6 sialoproteins significantly discriminated patients with OPC and oral cancer patients (p=0.0002 and p=0.0298, respectively).

Pearson's correlation analysis between salivary sialylation changes

Pearson's correlation analysis was performed to analyze the correlation between sialylation markers. As documented in Table 4, salivary sialidase activity exhibited significant positive correlation with TSA/TP ratio (r=0.597, p=0.003) and with α-2,6 ST activity (r=0.602, p=0.004). α-2,3 ST and α-2,6 ST also showed significant positive correlation (r=0.266, p=0.037).

Comparison of sialylation changes between pretreatment oral cancer patients and post-treatment follow-ups

Pretreatment (PT) salivary levels of sialylation endpoints in oral cancer patients were compared with their post treatment follow-ups values to examine their significance in treatment monitoring of oral cancer patients. The oral cancer patients were followed after anticancer treatment and were further divided into CR and NR as described in subjects and methods.

Figure. 6a depicted that the levels of TSA/TP ratio were comparable between PT and CR. While in case of NR the levels were elevated when compared to PT levels. The salivary sialidase activity was decreased in CR as compared to PT levels. While in case of NR the levels were found to be increased (Fig. 6a). As depicted in Fig. 6b, the levels of salivary α2-3 sialoproteins were found to be decreased in CR as compared to PT levels. While in case of NR, the levels were elevated as compared to PT levels. The levels of α2-6 sialoproteins were significantly decreased (p=0.026) in CR as compared to PT while levels were comparable between PT and NR. Moreover, α-2,3 and α-2,6 ST activities were found to be significantly decreased in CR (p=0.001 and 0.010, respectively) as compared to PT levels (Fig. 6c). α-2,3 ST activity was found to be increased in NR as compared to PT levels, while α-2,6 ST activity was comparable.

Discussion

The alterations in cell surface glycoconjugates are among the key molecular changes that occur in cancer cell during malignant transformation of a cell. Salivary diagnostics can prove to be a better non-invasive tool as compared to serum testing. The inclusion of controls and patients with OPC helps in monitoring early changes occurring during neoplastic transformation to oral cancer. In the present study, salivary TSA/TP ratio was found to be significantly higher in oral cancer patients as compared to controls. Also the levels were higher in patients with OPC as compared to controls. ROC curve analysis indicated that salivary TSA/TP ratio significantly discriminated controls and oral cancer patients, as well as patients with OPC and oral cancer patients. Earlier studies from our laboratory [24] along with other studies have indicated elevated salivary TSA levels in oral cancer [25–28, 36–38]. In the present study, salivary TSA/TP ratio was found

Table 4 Pearson's correlation analysis between salivary sialylation markers

Saliva	TSA/TP	Sialidase	α-2,3 sialoproteins	α-2,6 sialoproteins	α-2,3 ST	α-2,6 ST
TSA/TP		r=0.597 p=0.003	r=0.247 p=0.309	r=0.028 p=0.955	r=0.086 p=0.554	r=0.232 p=0.091
Sialidase	r=0.597 p=0.003		r=−0.512 p=0.299	r=−0.203 p=0.870	r=−0.257 p=0.304	r=0.602 p=0.004
α2-3 sialoproteins	r=0.247 p=0.309	r=−0.512 p=0.299		r=−0.176 p=0.676	r=0.030 p=0.907	r=0.018 p=0.943
α2-6 sialoproteins	r=0.021 p=0.955	r=−0.203 p=0.870	r=−0.176 p=0.676		r=0.262 p=0.465	r=0.110 p=0.733
α-2,3 ST	r=0.086 p=0.554	r=0.257 p=0.304	r=0.030 p=0.907	r=0.262 p=0.465		r=0.266 p=0.037
α-2,6 ST	r=0.232 p=0.091	r=0.602 p=0.004	r=0.018 p=0.943	r=0.110 p=0.733	r=0.266 p=0.037	

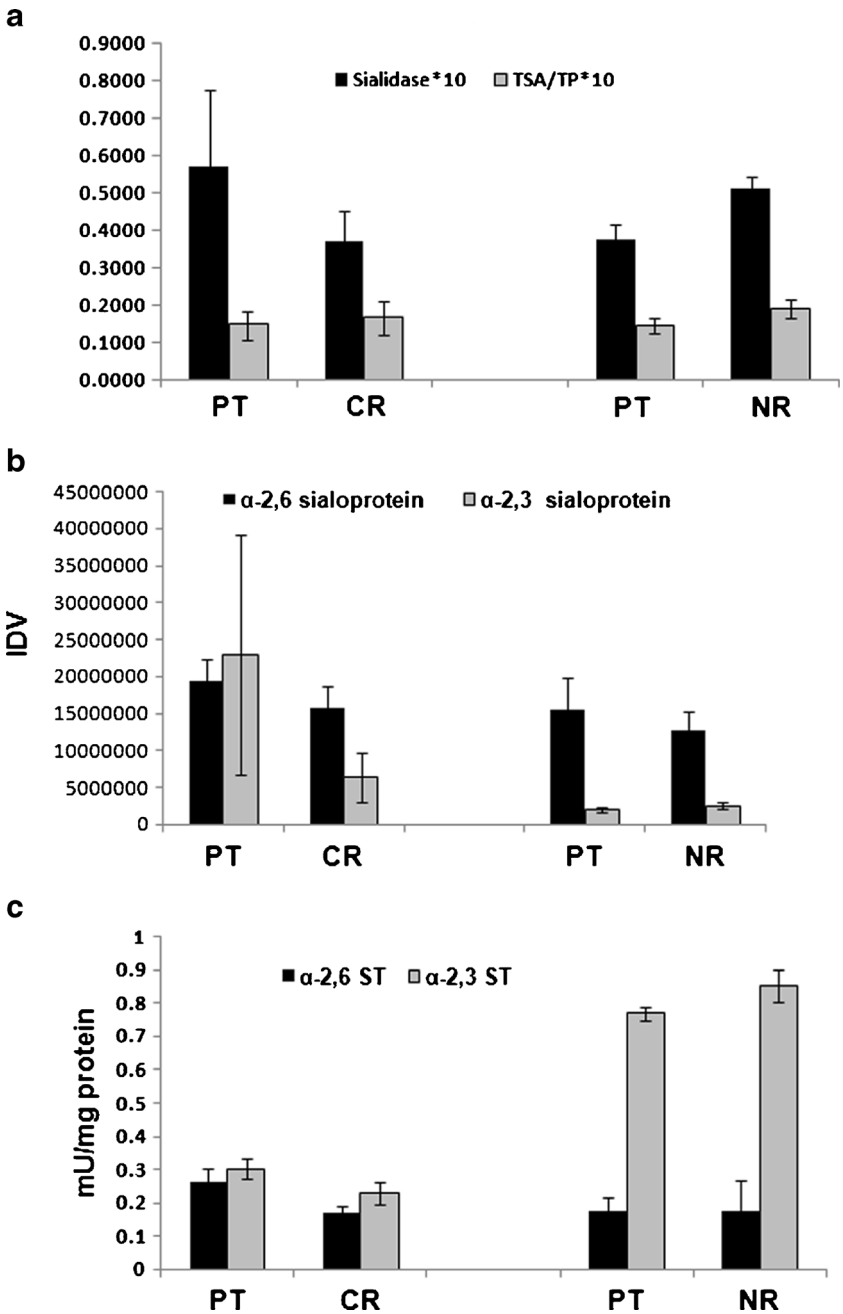

Fig. 6 Paired 't' test to assess the levels of sialylation markers in Pretreatment (PT), Complete responders (CR) and Non-responders (NR). (**a**) Comparison of sialidase activity in PT (N=15), CR (N=10) and NR (N=5) and TSA/TP ratio in PT (N=30), CR (N=25) and NR (N=5). (**b**) Comparison of α-2,3 and α-2,6 sialoproteins in PT (N=15), CR (N=10) and NR (N=5) (**c**) Comparison of α2-3 and α2-6 ST in PT (N=30), CR (N=25) and NR (N=5). *PT*, pretreatment; *CR*, complete responders; *NR*, non-responders; *ST*, sialyl transferase

to be higher in patients with LN metastasis when compared to patients without LN metastasis. An increasing trend was observed from controls to patients with early stage to advanced stage of disease. Earlier studies have reported salivary TSA as indicators for staging and tumor burden in oral cancer [11].

In the present study, significantly elevated levels of salivary sialidase activity were observed in oral cancer patients as compared to controls and patients with OPC. Also the levels were higher in patients with OPC as compared to the controls. ROC curve analysis depicted that salivary sialidase activity significantly distinguished controls and oral cancer patients as well as patients with OPC and oral cancer patients. Earlier studies have reported elevated levels of sialidase activity in oral secretions of patients with upper aerodigestive tract tumors [15]. Alterations in tissue and serum sialidase activity have been observed in various cancers [14, 16]. However, there are no earlier reports on evaluation of salivary sialidase activity in patients with OPC and oral cancer patients along with other sialylation changes. Pearson's correlation analysis depicted significant positive correlation between TSA and sialidase activity, which highlights that increase sialidase activity causes increase cleavage of sialic acid from sialoglycoconjugates.

Salivary α-2,6 and α-2,3 ST activities were found to be increased in patients with OPC and oral cancer patients as compared to the controls. Earlier studies from our laboratory have indicated alterations in α-2,6 and α-2,3 ST enzyme activity and *ST3GAL1* mRNA in oral cancer [7, 39]. However, there are no earlier reports of salivary linkage specific ST alterations in patients with OPC and oral cancer. In the present study, α-2,6 and α-2,3 ST activities were comparable between controls, early and advanced malignant disease, while both the enzyme activities were higher in patients with LN metastasis as compared to the patients without LN metastasis. Earlier studies from our laboratory have observed higher serum expression of α-2,6 and α-2,3 ST in patients with metastasis in oral cancer [7]. Other studies have reported higher tissue ST activity in metastasis of cervix and colon [40, 41].

A progressive increase in salivary α2-3 sialoproteins was observed from controls to patients with OPC to oral cancer patients. ROC curve analysis also revealed significant discriminatory efficacy of α2-3 sialoproteins in distinguishing controls and oral cancer patients as well as controls and patients with OPC. An increase of both the linkage specific sialoproteins was observed in advanced disease as compared to early disease. Previous studies from our laboratory have reported alterations in tissue and serum sialylated glycoproteins in oral cancer patients [7]. Various other investigators have documented alterations in sialylated glycoproteins in hepatocarcinoma and gliomas [42, 43]. However salivary estimation of α2-3 and α2-6 sialoproteins have not been reported earlier in patients with OPC and oral cancer patients.

To our knowledge simultaneous evaluation of salivary TSA, Sialidase activity, α-2,3 and α-2,6 ST activities, α2-3 and α2-6 sialoproteins have not been studied earlier in patients with OPC and oral cancer patients, which is a major strength of the present investigation.

The analysis of salivary molecular markers is the most effective tool for screening and follow-up of cancer patients during anticancer treatment. Earlier studies from out laboratory have observed significance of serum sialylation in treatment monitoring [7]. In the present study, salivary sialylation profile was also determined in follow-up oral cancer patients to assess its usefulness for treatment monitoring. The levels of TSA/TP ratio were comparable between PT and CR, while levels of sialidase activity, α-2,3 and α-2,6 sialoproteins and STs were decreased in CR as compared to PT levels. Sialidase activity, TSA/TP ratio, α-2,3 STs, and α-2,3 sialoproteins were increased in NR as compared to PT levels. Moreover, significantly lower levels of α2,3-sialoproteins in PT and NR samples were observed, while levels were markedly higher of α2.3-ST activity in these samples. We hypothesize that the differences might be due to increased sialidase activity. If sialidase activity is more in samples, then the expression of sialoproteins will be decreased even if ST activity is high. This might be due to increased break down of sialic acid from sialoproteins by sialidase enzyme. Hence, we assume that increase in sialidase activity might be much more than ST activity.

It was notable that marker levels increased prior to clinical detection of recurrence and decreased in patients with remission. It was evident that sialylation changes occur during anticancer treatment. Thus, close monitoring of salivary sialylation changes may be a promising approach to assess treatment outcome during post-treatment follow-up. The results provide an incentive for finding of newer drugs targeting sialylation.

In conclusion, the altered salivary sialylation changes might prove to be an effective non-invasive tool in monitoring early changes occurring during oral carcinogenesis, and predict disease progression and treatment response in oral cancer patients.

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Conflict of Interest None declared.

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